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Electrochemical detection of dipeptides and dipeptide amides

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ABSTRACT

A postcolumn reagent is used to create electroactive species from non-electroactive peptides. The reagent, based on the classical biuret reagent, consists of Cu(II), tartrate, bicarbonate and base. The detection is by dual electrode electrochemical detection. N-Acetylated dipeptides are oxidized at pH 12 and low potential. Dipeptides and carboxy terminal dipeptide amides give useful signals at lower pH. The dipeptide amides react with Cu(II) as do the tripeptides to yield the electronic spectrum and electrochemistry of the biuret complex. The complexes formed from the dipeptides are reversibly oxidized at potentials greater than 0.85 V *vs.* Ag/AgCl, 3 *M* NaCl. Analytically useful signals are obtained for the dipeptide amides at sensitivities equivalent to the sensitivities for longer peptides, 3–5 nA/μM, while the sensitivities for the dipeptides are about an order of magnitude lower.

INTRODUCTION

Recently[1] it has been shown that the classical biuret reaction[2] can be used in the electrochemical detection of peptides. The complex between Cu(II) and the peptide is formed in a weakly basic solution containing tartrate to stabilize the copper. The electrochemical activity results from the ease of oxidation of the Cu(II) complex thus formed[3]. The advantage of this procedure over other procedures that are based on amine specific reagent chemistry[4–10] or specific amino acid determination[11–19] are several. The amine specific reagents are not peptide selective. There are many more amines in human body fluids and other biological media than there are peptides. As a consequence a heavy burden is placed on chromatography for the required selectivity for trace peptide determinations. Furthermore, some peptides have no primary amine. Peptides that do not contain lysine and that have a pyroglutamate residue on the amine terminus or the amine terminus of which has been acetylated or formylated will not react with the primary and secondary amine specific reagents. Amino acid specific detection schemes can be very useful if the peptide sought is characterized by the presence of a particular amino acid. For example, the electrochemical detection of the opioid peptides[15] is conveniently carried out because of the presence of the oxidizable tyrosine residue. However, this approach suffers from the obvious problem that it is restricted to those peptides containing the detectable amino acid. The proce-

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dure using the biuret reagent is selective for the peptide bond. Detection limits of 0.25 pmol for small (3–6 amino acid-containing) peptides can be obtained using dual electrode electrochemical detection following the formation of the biuret complex in a postcolumn reactor.

In previous work[1] it was shown that the dipeptide glycyl–alanine (GA) was no more reactive than amino acids in this detection scheme, whereas the tripeptide glycyl–glycyl–glycine (G_3) was several thousand times more sensitively detected than non-electroactive amino acids. As a consequence, it would seem that, unfortunately, the selectivity of this detection scheme is restricted to tripeptides and longer peptides.

Margerum *et al.*[20] have carefully studied the influence of deprotonation of model copper peptide complexes on electrochemical activity of the complex. In Table I the data from Margerum's work have been abstracted into a convenient form. The table contains data for four derivatives of a simple tripeptide G_3. These are: the native peptide; the carboxyterminal amide; the N-formylated tripeptide; and the N-formylated carboxyterminal amide. The table shows that there is roughly an inverse relationship between the pH required to remove the last proton necessary for complex formation and the half-wave potential of the resulting complex. Thus, the simple tripeptide is fully formed in mild base (pH > 8.7) but requires a potential of 0.70 V (*vs.* Ag/AgCl, 3 *M* NaCl) for its oxidation. Contrarily, only at a pH > 12.1 would the biuret complex from the N-formylated carboxyterminal amide be fully formed, but its oxidation would occur at the relatively modest potential of 0.27 V. Intermediate are the N-formyl compound and the carboxyterminal amide.

The biuret reaction is given by dipeptide carboxyterminal amides (for example G–G amide)[20]. This work will show that the post-column reaction of dipeptide amides and dipeptides to form Cu(II) complexes and electrochemical detection of those complexes is possible for dipeptides and dipeptide amides with good sensitivity and only possible with acetylated dipeptides at extreme potentials and pH values.

EXPERIMENTAL

Reagents

The following reagents were used without further purification: potassium phosphate monobasic GR crystals (EM Science, Cherry Hill, NJ, U.S.A.), acetonitrile (HPLC grade) and phosphoric acid (EM Science), sodium carbonate anhydrous and

TABLE I

pK_{last} AND E° (*vs.* Ag/AgCl, 3 *M* NaCl) FOR G_3 DERIVATIVES

From ref. 20

Amine terminus	*Carboxyl terminus*			
	$-COO^-$		$-CONH_2$	
	pK_{last}	E°	pK_{last}	E°
H_2N-	6.72	0.70	8.69	0.42
HCOHN–	9.2	0.55	10.1	0.27

sodium bicarbonate (Fisher Scientific, Pittsburgh, PA, U.S.A.), sodium hydroxide pellets (J. T. Baker, Phillipsburgh, NJ, U.S.A.), L-α-Asp-L-Phe, L-α-Asp-L-Phe amide and L-Ala-L-Ala-L-Ala(A_3) (Sigma, St. Louis, MO, U.S.A.), Z-Gly-L-Phe (Z = carbobenzoxy), Z-Gly-L-Phe-NH_2, Gly-L-Phe and Gly-L-Phe-$NH_2 \cdot (CH_3COOH)$ (Peptide Institute, Japan), N-acetyl-Gly-L-Leu, N-Acetyl-Gly-L-Leu-NH_2, Gly-L-Leu and Gly-L-Leu-$NH_2 \cdot HCl$ (Research Plus, NJ, U.S.A.). Single letter abbreviations for the amino acids will be used hereafter: A = Ala, D = Asp, F = Phe, G = Gly, L = Leu. Potassium sodium tartrate (Aldrich, Milwaukee, WI, U.S.A.) was recrystallized from water before use. Water was doubly deionized and passed through an activated carbon bed before distillation in a Corning system.

Two reagent systems were used for chromatography. The mobile phase contained 25 m*M* KH_2PO_4 in acetonitrile–water (10:90) with added H_3PO_4 (around 2 g/l) to adjust the pH to 2.6. Postcolumn reagents contained 0.1 m*M* copper sulfate, 2 m*M* potassium sodium tartrate and 200 m*M* sodium bicarbonate except where mentioned. Sodium hydroxide and/or sodium carbonate were added in order to adjust to different pH values. These solutions were filtered through nylon 66 filters (pore size 0.2 μm, purchased from Rainin, Woburn, MA, U.S.A.) before use.

Instrumentation

A Waters Model 510 pump was used to pump the postcolumn reagents and a Waters Model M-45 was used to pump the mobile phase. These two pumps were operated by a Waters automated gradient controller in isocratic mode at a flow-rate of 2.0 ml/min. The mixing ratio was mobile phase–post-column reagents 40:60 (except where mentioned.)

A reversed-phase column, Waters Nova Pak-C_{18} was placed between the injector and mixing tee in the system. The detector was a glassy carbon dual electrode, Bioanalytical systems LC-17A electrochemical detector (W. Lafayette, IN, U.S.A.). A Bioanalytical system model LC-4B potentiostat was used to control the potential and measure the anode current. The cathode current-to-voltage converter was laboratory made. The pH was measured with an ORION Research pH meter and Fisher Scientific glass electrode.

RESULTS AND DISCUSSION

N-Terminal blocked dipeptides

An N-terminal blocked peptide, whether it is an amide, *e.g.* N-formyl, N-acetyl, pyroglutamyl (a lactam), or a carbamate, *e.g.* a carbobenzyloxy derivative, will have a less basic amine-terminal nitrogen than the native peptide. The three coordinating groups in an N-acetyl dipeptide are the N-terminal amide, the peptide amide and the terminal carboxyl group. An OH^- fills out the coordination sphere of Cu(II)[20]. The weakly basic amide is more acidic than the corresponding amine, and can be deprotonated to form the amido nitrogen ($RCO\bar{N}R'$) in the presence of base and Cu(II). In comparison to the amine, the amide requires a higher pH for complex formation (donor strength is $RCO\bar{N}R' > R\text{-}NH_2 > RCONHR'$), but once formed is easier to oxidize because the strongly donating amido nitrogen stabilizes the Cu(III).

The following compounds were chromatographed: N-acetyl-GL, N-acetyl-GL-NH_2, Z-GF, Z-GF-NH_2. The postcolumn reagent (using 50 m*M* $NaHCO_3$) was

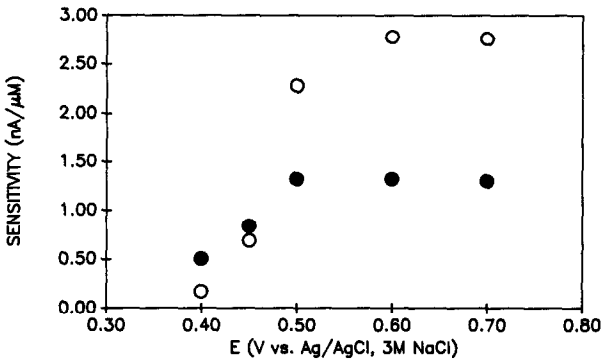

Fig. 1. Hydrodynamic voltammograms (anodic peak current *vs.* potential) for A_3(○) and N-acetyl-GL-NH_2(●). The sensitivities are calculated by dividing the peak current obtained by the injected concentration. Chromatographic conditions: total flow-rate, 1.7 ml/min; final pH = 12.7 ± 0.1; mobile phase [using 25 m*M* KH_2PO_4 in acetonitrile–water (10:90)]–postcolumn reagent (60:40) (using 0.2 m*M* Cu^{2+}, 4 m*M* tartrate 0.5 *M* $NaHCO_3$, 1.0 *M* NaOH). The injected volumes were 20 μl (containing 10 μ*M* A_3, 10 μ*M* N-acetyl-GL-NH_2 and 25 μ*M* N-acetyl-GL).

added with a volume fraction 0.5 to achieve a pH around 10.7–10.8, a volume fraction of 0.6 to achieve a pH of 11.6 ± 0.1 and a high pH and large buffer capacity postcolumn reagent (containing 0.5 *M* $NaHCO_3$, 1.0 *M* NaOH) was added with a volume fraction 0.4 (total flow-rate 1.7 ml/min) to achieve a pH of 12.7 ± 0.1. No signal was detected in the potential range 0.6–0.8 V for all but the N-acetyl-GL-NH_2. The sensitivity (nA/μ*M*) at the cathode for this compound was 0 at pH 10.7 and at pH 11.6 ± 0.1 was (potential) 0.005 (0.6), 0.003 (0.7), 0.017 (0.8). Hydrodynamic voltammograms for a control tripeptide, A_3, and N-acetyl-GL-NH_2 at pH 12.7 ± 0.01 are shown in Fig. 1. The collection efficiencies for all potentials were about 0.25. Even at this pH, there was no response from N-acetyl-GL, Z-GF-NH_2 and Z-GF. Thus, although it is possible to detect the N-terminal blocked dipeptide amides, it is not possible, with the current system, to determine N-terminal blocked dipeptides with a free carboxy terminus.

Free N-terminal dipeptides

Dipeptide amides yield the biuret reaction[20]. The coordinating groups are the free amino, two amide nitrogens and a hydroxide. The complex between GG-NH_2 and Cu(II) can be formed at a pH of 9.2, and is oxidizable with an E° of 0.67[20]. Other dipeptide-based compounds that give the biuret reaction have not been reported, to our knowledge. The simple dipeptides do react with Cu(II). For example DF, in a solution containing an excess of Cu(II) in basic tartrate, yields a broad absorbance band with $\lambda_{max} \approx 660$ nm. After heating (*ca.* 70°C, *ca.* 15 min) the band sharpens and increases in intensity, demonstrating a $\lambda_{max} \approx 640$ nm. If the donor ligands are $-NH_2$, amido, carboxylate and hydroxide, one would predict[20] a λ_{max} around 610 nm. The biuret reaction, as exemplified by the complex formed between DF-NH_2 and Cu(II), has a $\lambda_{max} \approx 560$ nm. Thus, the dipeptide coordinates with the Cu(II), but with two of the four ligands, carboxylate and hydroxide, not being significant stabilizers of the Cu(III) oxidation state, the complex may be difficult to oxidize.

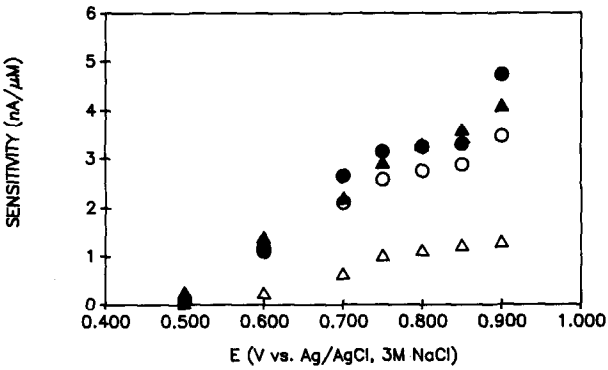

Fig. 2. Hydrodynamic voltammograms for A_3(○), GF-NH_2(●), GL-NH_2(△) and DF-NH_2(▲). Chromatographic conditions: total flow-rate 2.0 ml/min; final pH = 10.0 ± 0.1; mobile phase [using 25 m*M* KH_2PO_4 in acetonitrile-water (10:90)]–postcolumn reagent (40:60) (containing 0.1 m*M* $CuSO_4$, 2 m*M* tartrate, 0.2 *M* $NaHO_3$, 0.15 *M* NaOH). The injected volumes were 20 μl. Injections of dipeptide amides were of 5 μ*M* solutions and 25 μ*M* solutions of dipeptides were used. All injections contained 5 μ*M* A_3.

Hydrodynamic voltammograms for a control tripeptide, A_3, and the dipeptide amides DF-NH_2, GF-NH_2, GL-NH_2 are shown in Fig. 2, and those for the dipeptides GF, DF and GL are shown in Fig. 3. The half wave potential for the dipeptide amides are the same as for A_3, about 0.65 V. Also the sensitivities for three of the four compounds are similar. The sensitivity for GL-NH_2 is significantly lower than others, which indicates an unusual behavior. However, at 0.90 V a very large peak appears in the anodic chromatogram, but not in the cathodic chromatogram of GL-NH_2. There is obviously an impurity in the GL-NH_2 solid.

The dipeptides give analytically useful signals at 0.9 V, but not at lower potentials. The shift of $E_{1/2}$ to a higher potential has been discussed in terms of the ligands. At such high potentials, the background problem becomes significant at the anode, but the behavior at the cathode is better.

The sensitivities shown in Fig. 2 are somewhat lower than those reported earlier[1]; 8 nA/μ*M* at the anode. The lower values (3–5 nA/μ*M*) reported here are the result

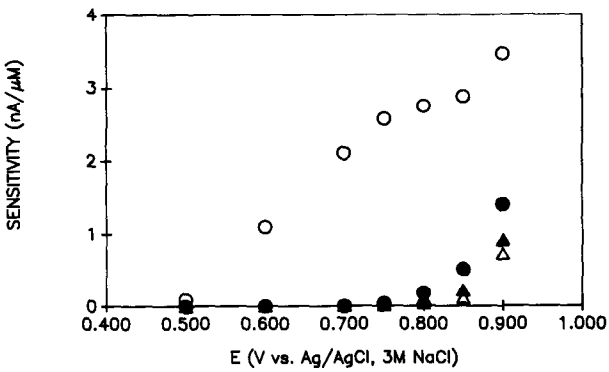

Fig. 3. Hydrodynamic voltammograms for A_3(○), GF (●), GL (△) and DF(▲). The chromatographic conditions are the same as in Fig. 2.

TABLE II

COLLECTION EFFICIENCIES

The chromatographic conditions are the same as in Fig. 2.

Peptide	*Potential*						
	0.5	*0.6*	*0.7*	*0.75*	*0.8*	*0.85*	*0.9*
A_3	–	0.24	0.25	0.27	0.28	0.28	0.24
GF-NH_2	–	0.24	0.26	0.29	0.27	0.26	0.15
GL-NH_2	–	0.25	0.27	0.27	0.27	0.26	0.24
DF-NH_2	0.29	0.30	0.31	0.30	0.31	0.31	0.30
GF	–	–	–	0.11	0.11	0.11	0.08
GL	–	–	–	–	0.02	0.10	0.09
DF	–	–	–	0.12	0.12	0.17	0.19

of greater dilution of the mobile phase by the postcolumn reagent in the current experiments. Although a variety of ratios have been used, they have been approximately mobile phase–postcolumn reagent (50:50). With the previous pressure-driven apparatus the ratio was about 9:1. By using a more concentrated reagent a lower mixing ratio could be used, with a resulting small increase in sensitivity.

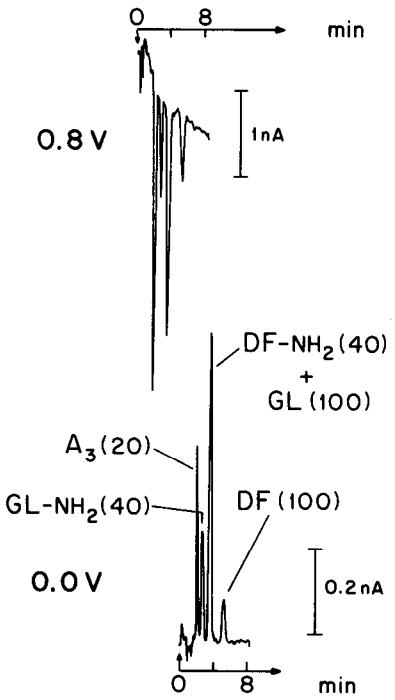

Fig. 4. A chromatogram of peptides. Mobile phase–postcolumn reagent ratio (40:60), flow-rate is 2.0 ml/min. The peaks (masses in pg) are A_3 (20); GL-NH_2 (40); DF-NH_2 and GL (40 and 100); DF (100). The top trace is the response of the anode and the bottom one is the response of the cathode.

Collection efficiencies have been determined for the analytes discussed. The collection efficiencies, ratios of cathodic to anodic current, should be around 0.35 for the geometry of this electrode system[1] if the oxidized product is perfectly stable. The values observed in Table II for A_3 and the amides are within the range of values previously seen for a variety of larger peptides, 0.25–0.30[1]. The collection efficiencies for the dipeptides are markedly lower and somewhat sensitive to potential.

The combination of lower anodic current and lower collection efficiency, the former by about a factor of four and the latter by about a factor of two to three, makes cathodic detection of the dipeptides about an order of magnitude less sensitive than the detection of higher peptides.

A chromatogram of peptides is shown in Fig. 4, the top trace is the response of the anode and the bottom one is the response of the cathode. This mixture of dipeptides, derivatives and the tripeptide was separated with a flow-rate of 0.8 ml/min [KH_2PO_4 in acetonitrile–water (10:90), pH 2.6 ± 0.1] and was mixed with 1.2 ml/min postcolumn reagent (containing 0.1 m*M* $CuSO_4$, 2 m*M* tartrate, 50 m*M* $NaHCO_3$, 100 m*M* Na_2CO_3, pH = 10.10 ± 0.1). The final pH was 9.7.

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